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(54) Title: BIOCONJUGATION OF OLIGONUCLEOTIDES

(57) Abstract

This invention discloses a novel method for conjugating RNA oligonucleotides with other molecular entities exclusively at the 5'–position of the oligonucleotide. Specifically, this invention discloses a method for conjugating or derivatizing RNA oligonucleotides via transcription in the presence of a 5'–modified guanosine. Included in the invention are the novel bioconjugated oligonucleotides that can be prepared according to the method of the invention.

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BIOCONJUGATION OF OLIGONUCLEOTIDES

FIELD OF THE INVENTION

This invention describes a novel method for conjugating oligonucleotides to other molecular entities exclusively at the 5'-position of the oligonucleotide. The method of this invention takes advantage of an enzymatic method of synthesizing RNA via an RNA polymerase.

BACKGROUND OF THE INVENTION

A method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules has been developed. This method, Systematic Evolution of Ligands by Exponential Enrichment, termed SELEX, is described in United States Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by Exponential Enrichment," now abandoned; United States Patent Application Serial No. 07/714,131, filed June 10, 1991, entitled "Nucleic Acid Ligands," now United States Patent No. 5,475,096; United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled "Methods for Identifying Nucleic Acid Ligands," now United States Patent No. 5,270,163 (see also, WO 91/19813), each of which is specifically incorporated by reference herein. Each of these applications, collectively referred to herein as the SELEX Patent Applications, describes a fundamentally novel method for making a nucleic acid ligand to any desired target molecule. The SELEX process provides a class of products which are referred to as nucleic acid ligands (also referred to in the art as "aptamers"), each ligand having a unique sequence and property of binding specifically to a desired target compound or molecule.

The SELEX method involves selection from a mixture of candidate oligonucleotides and step-wise iterations of binding, partitioning and amplification, using the same general selection scheme, to achieve virtually any desired criterion of binding affinity and selectivity. Starting from a mixture of nucleic acids, preferably comprising a segment of randomized sequence, the SELEX method includes steps of contacting the mixture with the target under conditions favorable for binding, partitioning unbound nucleic acids from those nucleic acids which have bound specifically to target molecules, dissociating the nucleic acid-target complexes,

amplifying the nucleic acids dissociated from the nucleic acid-target complexes to yield a ligand-enriched mixture of nucleic acids, then reiterating the steps of binding, partitioning, dissociating and amplifying through as many cycles as desired to yield highly specific high affinity nucleic acid ligands to the target molecule.

The basic SELEX method has been modified to achieve a number of specific objectives. For example, United States Patent Application Serial No. 07/960,093, filed October 14, 1992, entitled "Method for Selecting Nucleic Acids on the Basis of Structure," abandoned in favor of United States Patent Application Serial No. 08/198,670, describes the use of SELEX in conjunction with gel electrophoresis to select nucleic acid molecules with specific structural characteristics, such as bent DNA. United States Patent Application Serial No. 08/123,935, filed September 17, 1993, entitled "Photoselection of Nucleic Acid Ligands," describes a SELEX based method for selecting nucleic acid ligands containing photoreactive groups capable of binding and/or photocrosslinking and/or photoinactiviating a target molecule. United States Patent Application Serial No. 08/134,028, filed October 7, 1993, entitled "High-Affinity Nucleic Acid Ligands That Discriminate Between Theophylline and Caffeine," abandoned in favor of United States Patent Application Serial No. 08/443,957, now United States Patent No. 5,580,737, describes a method for identifying highly specific nucleic acid ligands able to discriminate between closely related molecules, which can be non-peptidic, termed Counter-SELEX. United States Patent Application Serial No. 08/143,564, filed October 25, 1993, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Solution SELEX," abandoned in favor of United States Patent Application Serial No. 08/461,069, now United States Patent No. 5,567,588, describes a SELEX-based method which achieves highly efficient partitioning between oligonucleotides having high and low affinity for a target molecule. United States Patent Application Serial No. 08/434,425, filed May 3,1995, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Tissue SELEX" and United States Patent Application Serial No. 08/433,124, filed May 3, 1995, entitled "Nucleic Acid Ligands of Tissue Target," describe the use of SELEX to identify and prepare Nucleic Acid ligands to Tissue Targets.

The SELEX method encompasses the identification of high-affinity nucleic acid ligands containing modified nucleotides conferring improved characteristics on the ligand, such as improved *in vivo* stability or improved delivery characteristics. Examples of such modifications include chemical substitutions at the ribose and/or phosphate and/or base positions. SELEX-identified nucleic acid ligands containing modified nucleotides are described in United States Patent Application Serial No. 08/117,991, filed September 8, 1993, entitled "High Affinity Nucleic Acid Ligands containing Modified Nucleotides," abandoned in favor of United States Patent Application Serial No. 08/430,709, now United States Patent No. 5,660,985, that describes oligonucleotides containing nucleotide derivatives chemically modified at the 5- and 2'-positions of pyrimidines. United States Patcnt Application Serial No. 09/134,028, *supra*, describes highly specific nucleic acid ligands containing one or more nucleotides modified with 2'-amino (2'-NH_2), 2'-fluoro (2'-F), and/or 2'-O-methyl (2'-OMe). United States Patent Application Serial No. 08/264,029, filed June 22, 1994, entitled "Novel Method of Preparation of Known and Novel 2'-Modified Nucleosides by Intramolecular Nucleophilic Displacement," describes oligonucleotides containing various 2'-modified pyrimidines.

The SELEX method encompasses combining selected oligonucleotides with other selected oligonucleotides and non-oligonucleotide functional units as described in United States Patent Application Serial No. 08/284,063, filed August 2, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Chimeric SELEX," now United States Patent No. 5,637,459 and United States Patent Application Serial No. 08/234,997, filed April 28, 1994, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," now United States Patent No. 5,683,867, respectively. These applications allow the combination of the broad array of shapes and other properties, and the efficient amplification and replication properties, of oligonucleotides with the desirable properties of other molecules.

The SELEX method encompasses complexes of oligonucleotides. United States Patent Application Serial No. 08/434,465, filed May 4, 1995 entitled "Nucleic Acid Ligand Complexes," describes a method for preparing a therapeutic or

diagnostic complex comprised of a nucleic acid ligand and a lipophilic compound or a non-immunogenic, high molecular weight compound.

Nucleic acid ligands derived by the SELEX process have been used in diagnostic applications. (See e.g., United Stated Patent Application No. 08/487,425, filed June 7, 1995, entitled "Enzyme Linked Oligonucleotide Assays (ELONAS)," United States Patent Application No. 08/479,729, filed June 7, 1995, entitled "Use of Nucleic Acid Ligands in Flow Cytometry," and United States Patent Application No. 08/628,356, filed April 5, 1996, entitled "Method for Detecting a Target Compound in a Substance Using a Nucleic Acid Ligand." The full text of the above described patent applications, including but not limited to, all definitions and descriptions of the SELEX process, are specifically incorporated by reference herein in their entirety.

Considerable research is being directed to the application of oligonucleotides and oligonucleotide analogs as diagnostic and research reagents and as potential therapeutic agents. There are currently at least three areas of exploration regarding the use of oligonucleotides as pharmaceutical compounds. In the most advanced field, antisense oligonucleotides are used to bind to certain coding regions in an organism to prevent the expression of proteins or to block various cell functions. Additionally, the discovery of RNA species with catalytic functions -- ribozymes -- has led to the study of RNA species that serve to perform intracellular reactions that will achieve desired effects. And lastly, the discovery of the SELEX process (Systematic Evolution of Ligands by Exponential Enrichment) (Tuerk and Gold (1990) Science 249:505) has shown that oligonucleotides can be identified that will bind to almost any biologically interesting target.

The use of antisense oligonucleotides as a means for controlling gene expression and the potential for using oligonucleotides as possible pharmaceutical agents has prompted investigations into the introduction of a number of chemical modifications into oligonucleotides to increase their therapeutic activity and stability. Such modifications are designed to increase cell penetration of the oligonucleotides, to stabilize them from nucleases and other enzymes that degrade or interfere with the structure or activity of the oligonucleotide analogs in the body, to enhance their binding to targeted RNA, to provide a mode of disruption (terminating event) once

sequence-specifically bound to targeted RNA and/or to improve the pharmacokinetic properties of the oligonucleotides.

Recent research has shown that RNA secondary and tertiary structures can have important biological functions (Tinoco *et al.* (1987) Cold Spring Harb. Symp. Quant. Biol. 52:135; Larson *et al.* (1987) Mol. Cell. Biochem. 74:5; Tuerk *et al.* (1988) Proc. Natl. Acad. Sci. USA 85:1364; Resnekov *et al.* (1989) J. Biol. Chem. 264:9953). PCT Patent Application Publication WO 91/14436, entitled "Reagents and Methods for Modulating Gene Expression Through RNA Mimicry," describes oligonucleotides or oligonucleotide analogs which mimic a portion of RNA able to interact with one or more proteins. The oligonucleotides contain modified internucleoside linkages rendering them nuclease-resistant, have enhanced ability to penetrate cells, and are capable of binding target oligonucleotide sequences.

The use of oligonucleotides as therapeutic and diagnostic agents is growing rapidly with many compounds in preclinical and human clinical trials. In many of these applications the oligonucleotide is derivatized or conjugated with another molecular entity. These conjugations are typically performed for the purpose of attaching fluorescent dyes or other diagnostic reporter groups or for attaching compounds that modulate the activity or the pharmacokinetic behavior of the oligonucleotide. For example, Smith *et al.* describe the synthesis of fluorescent dye-conjugated primers for use in fluorescence-based DNA sequence analysis (Smith *et al.* (1987) Methods Enzymol. 155: 260-301). United States Patent No. 5,650,275 of Pitner *et al.*, describes the use of spectroscopically detectable labeled nucleic acid ligands to determine the presence or absence of a target compound in a sample (see also copending United Stated Patent Application No. 08/487,425, filed June 7, 1995, entitled "Enzyme Linked Oligonucleotide Assays (ELONAS)," United States Patent Application No. 08/479,729, filed June 7, 1995, entitled "Use of Nucleic Acid Ligands in Flow Cytometry," and United States Patent Application No. 08/628,356, filed April 5, 1996, entitled "Method for Detecting a Target Compound in a Substance Using a Nucleic Acid Ligand"). United States Patent Application Serial No. 08/434,465, filed May 4, 1995, entitled "Nucleic Acid Ligand Complexes," describes the use of oligonucleotides conjugated to lipophilic compounds or non-

immunogenic, high molecular weight compounds to modulate the activity or pharmokinetic behavior of the oligonucleotides. A lipophilic compound covalently attached to an antisense oligonucleotide through a phosphoester bond has been described in EP 462 145 B1 of Bischofberger. Conjugation has also been used to make oligonucleotide dimers and to attach oligonucleotides to multimeric platforms. (Jones *et al.* (1995) J. Med. Chem. 38:2138).

Several chemical methods exist to accomplish such conjugations. (For a review, see Goodchild (1990) Bioconjugate Chemistry 1:165-187). The presence of a chemically reactive functional group, such as an amine or thiol, at the 5'-terminus of an oligonucleotide allows selective attachment of various conjugates, including reporter groups (Smith *et al.* (1987) Methods Enzymol. 155:260-301; Sproat *et al.* (1987) Nucleic Acids Res. 15:6181-6196) and peptide epitopes (Tung *et al.* (1991) Bioconjugate Chem. 2:464-465; Bruick *et al.* (1997) Chem. Biol. 3:39-56). Oligodeoxynucleotides containing a terminal amino functionality have been utilized for the construction of bioconjugates with novel properties. In some of the more common methods of synthesizing these bioconjugates, a primary aliphatic amine group is incorporated at the 5'-terminus of the oligonucleotide in the final step of the assembly of a synthetic oligonucleotide (Tung *et al.* (1991) Bioconjugate Chem. 2:464-465; Smith *et al.* (1987) Methods Enzymol. 155:260-301). A commercial reagent (actually a series of such linkers having various lengths of polymethylene connectors) for linking to the 5' terminus of an oligonucleotide is 5'-Amino-Modifier C6. These reagents are available from Glen Research Corp (Sterling, VA). These compounds have been used by Krieg (Krieg *et al.* (1971) Antisense Res. and Dev.1:161) to link fluorescein to the 5'-terminus of an oligonucleotide. Since many macromolecules of interest are hydrophilic, these reactions are generally done in water, requiring large excesses of reagent to overcome the competing hydrolysis. Usually the amine on the oligonucleotide is added to the terminus of the molecule and must compete with free amine and alcohol on the fully deprotected oligonucleotide if this modification is done post-synthetically.

In another common method of conjugating oligonucleotides to other molecular entities, particularly detector molecules, the molecular entity is converted

into a phosphoramidite, which is then added to the free alcohol of the full length oligonucleotide which is attached to a solid support. This method is less than ideal due to the air and water sensitivity of the phosphoramidite, as well as the fact that the molecule can only be added to the terminus of the oligonucleotide. Furthermore, many detector molecules are not compatible with this method due to the harsh conditions normally needed to fully deprotect and release the oligonucleotide from the support. A third method of conjugating oligonucleotides to other molecules is the coupling of an alkylthio derivatized oligonucleotide with a α-haloacetyl or with a maleimide containing compound. (Jones *et al.* (1995) J. Med. Chem. 38:2138).

An alternative method for the synthesis of oligodeoxynucleotides terminated by 5'-amino-5'-deoxythymidine has been described (Bruick *et al.* (1997) Nucleic Acids Res. 25:1309-1310). This method uses a DNA template to direct the ligation of a peptide to an oligonucleotide, in which the peptide is presented by a second oligonucleotide in the form of a reactive thioester-linked intermediate.

Oligodeoxynucleotides have been labeled for potential *in vivo* diagnostic imaging by two methods. Hnatowich has synthesized oligodeoxynucleotides with a primary amine on the 5'-terminus then coupled peptidyl Tc chelates via NHS chemistry (Hnatowich (1995) J. Nucl. Med. 36:2306). Hayes *et al.* have synthesized 5'-[fluorenylmethoxycarbonyl]-5-(E)-[2-tri-n-butylstannylvinyl]-2'-deoxyuridine-3'-cyanoethyl N,N-diisopropylphosphoramidite). (Hayes *et al.* (1997) Nucleic Acid Res. 25:2897-2901). This reagent is compatible with automated solid-phase synthesis and has been incorporated into the thrombin aptamer. The modified deoxynucleotide is readily iodinated with ^{123}I as a potential thrombus imaging agent. Both of these techniques apply to synthetic deoxyoligonucleotides and are not transferable to ribooligonucleotides produced synthetically.

Conjugates of oligonucleotides with peptides having specific functions can be useful for various applications. Examples include the use of a nuclear transport signal peptide to direct intracellular trafficking (Eritja *et al.* (1991) Tetrahedron 47: 4113-4120); a hydrophobic peptide (Juby *et al.* (1991) Tetrahedron Lett. 32:879-822) or polylysine (Leonetti *et al.* (1991) Bioconjugate Chem. 1:149-153) to increase cell penetrability, and polylysine to provide multiple attachment sites for nonradioactive

reporting probes (Haralambidis *et al.* (1987) Tetrahedron Lett. 28:5199-5202; Haralambidis *et al.* (1990) Nucleic Acids Res. 18:493-499).

Transcription from synthetic DNA templates using T7 RNA polymerase is a convenient method for the synthesis of RNA oligonucleotides. The transcription of DNA by T7 RNA polymerase begins at a uniquely defined base relative to the promoter DNA sequence (Chamberlin and Ring (1973) J. Biol. Chem. 248:2235-2244). The first nucleotide transcribed is usually a purine. The transcription of a DNA template into an RNA is distinct in that it results in a new RNA having a triphosphate at its 5' terminus. The effects of modifying the 5'-position in the initiating nucleotide in RNA synthesis was studied by Martin and Coleman. (Martin and Coleman(1989) Biochemistry 28:2760-2762). Martin and Coleman discovered that the first nucleotide incorporated into an RNA transcript is unique in that the 5'-triphosphate is not utilized in a bond-formation step. That is, while Watson/Crick base-pairing is involved, the 5' region of the initial nucleotide is not involved in binding to the protein and/or to the DNA template. Thus, it was observed that initiation of DNA transcription by T7 RNA polymerase proceeds effectively whether initiated with guanosine triphosphate (GTP), guanosine monophosphate (GMP) or guanosine.

The use of the modified guanosine, 5'-amino-5'-deoxyguanosine, as an initiator in enzymatic RNA synthesis has been described by Lohse and Szostak. (Lohse and Szostak (1996) Nature 381:442-444). Synthesis of this molecule is difficult.

To date, the use of a modified guanosine as an initiator in enzymatic RNA synthesis, wherein the guanosine has a substituent at the 5'-position that is larger than a triphosphate, has not been demonstrated.

SUMMARY OF THE INVENTION

The present invention describes a novel and highly efficient method for derivatizing or conjugating oligonucleotides with other molecular entities. Specifically, the present invention describes a method for enzymatically generating oligonucleotides derivatized exclusively at the 5'-position of the oligonucleotide,

using 5'-substituted guanosines as initiators in the enzymatic synthesis of RNA. The methods disclosed herein allow for the addition of a variety of molecular entities -- including but not limited to reactive molecules, reporter molecules, reporter enzymes, lipophilic molecules, peptides and proteins -- to the 5'-terminus of nascent RNA oligonucleotides.

In its most basic form the method of the instant can be described by the following steps:

a) providing a DNA template; and b) combining the DNA template with nucleotide triphosphates, a 5'-substituted guanosine and an RNA polymerase under conditions suitable for transcription. In a preferred embodiment the initiating base on the RNA is a guanosine and the RNA polymerase is T7 RNA polymerase. The types of nucleotide triphosphates used will depend on the composition of the template and the desired RNA product.

The method of this invention utilizes a 5'-modified guanosine monophosphate (GAP) as the initiator in an RNA polymerase-catalyzed template-dependent transcription. The guanosine initiator is modified at the 5'-position with a molecular entity whose chemical nature is compatible with RNA transcription. These guanosines can be substituted at the 5'-position with molecular entities which differ greatly in size from the triphosphate group of a guanosine triphosphate. Examples of molecular entities that may be coupled to the oligonucleotide include, but are not limited to other macromolecules, such as oligonucleotides, lipophilic compounds, such as cholesterol, phospholipids, diacyl glycerols and dialkly glycerols, proteins, peptides or carbohydrates, polymers or resins, such as polystyrene, diagnostic detector molecules, such as biotin or fluorescein, reporter enzymes, photoaffinity labels, steroids, pharmacokinetic modulators such as PEG, lipids or liposomes, reactive moieties for post-transcriptional conjugation such as a hexylamine or a diene or dienophile, and chelates for binding metals.

The molecular entity can be designed to serve in a large variety of functions. For example, a reporter group such as biotin or a fluorescent molecule may be incorporated into the bioconjugate to provide reporter bioconjugates for use as diagnostic reagents. A macromolecule such as a polyethylene glycol may be

incorporated into the bioconjugate to provide a bioconjugate with improved pharmacokinetics. Chelates for binding metals, particularly radioactive metals such as ^{99}m Tc can be attached to the oligonucleotide for diagnostic imaging purposes. Other radioactive metals, such as rhenium-188, can be conjugated for directed radiotherapy applications. Bioconjugates may also comprise peptides which are reactive to an active site on a protein. Other labeling haptens, such as the Bolton-Hunter reagent can be incorporated to facilitate radio-iodination. Structural probes such as fluorescent quenching agents or spin labels can be incorporated to study protein-nucleic acid interactions. To facilitate covalent SELEX a photoaffinity label, such as a psoralen, acridine, or a like molecule can be conjugated. A chemical entity such as a diene or Schiffs base could be incorporated for chemical covalent SELEX. In an embodiment of parallel SELEX the combinatorial small molecule library can be conjugated to the transcript. The molecular entity can also be designed to serve as a photoaffinity label. Histological probes may be incorporated into the bioconjugate for visualization or anitbody staining.

This application further discloses a method for generating bioconjugates comprising nucleic acid ligands derivatized with a molecular entity exclusively at the 5'-position of the nucleic acid ligands. This particular embodiment takes advantage of the method for identifying nucleic acid ligands referred to as SELEX, an acronym for Systematic Evolution of Ligands by EXponential enrichment.

Briefly, bioconjugates to a target are identified by the SELEX method by the steps comprising:

1) preparing a candidate mixture of bioconjugates by the steps comprising (a) providing a DNA template having a sequence to be transcribed and (b) combining the DNA template with nucleotide triphosphates, a 5'-modified guanosine, and an RNA polymerase under conditions suitable for transcription;

2) contacting the bioconjugate candidate mixture with a target, wherein bioconjugates having an increased affinity to the target relative to the bioconjugate candidate mixture may be partitioned from the remainder of the bioconjugate candidate mixture;

3) partitioning the increased affinity bioconjugates from the remainder of the bioconjugate candidate mixture; and

4) amplifying the increased affinity bioconjugates to yield a ligand-enriched mixture of bioconjugates, whereby bioconjugates of the target are identified.

The 5'-substituted GAP can aid in (1) the SELEX partition step, e.g. BIA-SELEX (see United States Application Serial No. 08/792,075, filed January 31, 1997, entitled "Flow Cell SELEX," which is incorporated herein by reference), plate SELEX (Conrad *et al.* (1996) Methods of Enzymol. 267:336), and streptavidin column partitions, (2) monitoring the progress of a SELEX using a reporter substitution, and/or (3) participating directly with the target protein, e.g., Blended SELEX (United States Patent No. 5,683,867, issued November 04, 1997, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX").

One embodiment of this invention is an extension of the Blended SELEX methodology (United States Patent No. 5,683,867, issued November 04, 1997, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX," which is incorporated herein by reference in its entirety), providing a novel means for identifying and generating oligonucleotides with specifically selected properties. This embodiment of the invention provides a method for identifying and synthesizing oligonucleotides derivatized with molecular entities, which are selected based upon the desired properties for the oligonucleotide, examples of which are described above.

In another embodiment of this invention the 5'-derivatized guanosine contains a reactive moiety which can be used for post-transcription conjugation of the transcript. This embodiment of the invention can be described by the following steps: a) providing a DNA template b) combining the DNA template with nucleotide triphosphates, a 5'-substituted guanosine, wherein said 5'-substituent contains a reactive moiety and an RNA polymerase under conditions suitable for transcription; and c) reacting the product from step b) with a molecular entity containing a moiety capable of reacting with the reactive moiety on said 5'-substituent. Post transcription conjugation is necessary to obtain oligonucleotides derivatized with molecular entities which are not compatible with transcription.

Examples of reactive moieties include but are not limited to amines, dienes, dienophiles, thiols, vinylsulfones, photoaffinity labels and interchelators.

Also included in this invention are any novel conjugated oligonucleotides which can be produced by the method of this invention.

BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1 illustrates graphically the percent incorporation of GAP and the yield of GAP transcript at concentrations of GAP in the range between 0 and 10 mM.

FIGURE 2 depicts the results of the GAP-TEG-biotin / γ-^{32}P-GTP initiation competition assay described in Example 2. The products of the transcription reactions were analyzed by denaturing gel electrophoresis. All lanes are labeled with the ratio of GAP-biotin to GTP, with the exception of lane C which contains 6 mM GTP as a control. As the concentration of GAP-biotin increased the γ-^{32}P-GTP decreased.

FIGURE 3 shows the results of the Streptavidin shift assay described in Example 2. The reaction products were combined with Streptavidin and analyzed by denaturing gel electrophoresis. All lanes are labeled with the ratio of GAP-biotin to GTP.

FIGURE 4A shows the results of the transcription reactions with GAP analogs **11-16**. Lanes 1 and 2 contain the transcript without GAP, lane 3 is the GAP-TEG transcript, lane 4 is the GAP-biotin transcript, lane 5 is the GAP-TEG-biotin transcript, lane 6 is the GAP-Tc chelate transcript, lane 7 is the GAP-TEG-Tc chelate transcipt, lane 8 is the GAP-fluorescein transcript and lane 9 is the GAP-TEG-fluorescein transcript. The analysis was performed on an 8% polyacrylamide gel containing 7 M urea.

FIGURE 4B depicts the transillumination of the fluorescein-GAP initiated transcripts in lanes 8 and 9.

FIGURE 5 shows the results of the ^{99}m Tc labeling of the GAP-Tc chelate initiated transcript. The ^{99}m Tc labeled transcript was analyzed by 8% polyacrylamide denaturing gel electrophoresis in the absence of EDTA. Lane 1 contains the ^{32}P full length transcript control and lane 2 contains the ^{99}m Tc labeled GAP-Tc chelate.

DETAILED DESCRIPTION OF THE INVENTION

The present invention includes a novel method for enzymatically generating oligonucleotide bioconjugates. Specifically, this invention describes a novel method for enzymatically generating bioconjugates comprising RNA oligonucleotides derivatized specifically at the 5'-position with a molecular entity. This method utilizes 5'-substituted guanosines as initiators in RNA polymerase catalyzed template-directed synthesis of bioconjugates. The method of this invention can be used to conjugate an oligonucleotide prior to transcription or to incorporate a reactive moiety into the transcript which can then be used to bioconjugate the oligonucleotide post-transcription. This method may be applied to the synthesis of a variety of conjugated ribonucleotides including nucleic acid ligands, ribozymes and antisense RNA.

The molecular entity can be any molecule, including another macromolecule, which is compatible with transcription. Examples of molecular entities that may be coupled to the oligonucleotide include, but are not limited to other macromolecules, such as oligonucleotides, lipophilic compounds, proteins, peptides or carbohydrates, polymers or resins, such as polystyrene, diagnostic detector molecules, such as biotin or fluorescein, reporter enzymes, photoaffinity labels, steroids, pharmacokinetic modulators such as PEG, lipids or liposomes, reactive moieties for post-transcriptional conjugation such as a hexylamine or a diene or dienophile, and chelates for binding metals.

The molecular entity can be designed to serve in a large variety of functions. For example, a reporter group such as biotin or a fluorescent molecule may be

incorporated into the bioconjugate to provide reporter bioconjugates for use as diagnostic reagents. A macromolecule such as a polyethylene glycol may be incorporated into the bioconjugate to provide a bioconjugate with improved pharmacokinetics. Chelates for binding metals, particularly radioactive metals such as ^{99}m Tc can be attached to the oligonucleotide for diagnostic imaging purposes. Other radioactive metals, such a rhenium-188, can be conjugated for directed radiotherapy applications. Bioconjugates may also comprise peptides which are reactive to an active site on a protein. Bioconjugates can also be used to attach the oligonucleotide to columns, solid support matrices, or surfaces such as microtiter plates.

Certain terms used to describe the invention hercin are defined as follows:

"**Nucleoside**" means either a deoxyribonucleoside or a ribonucleoside or any chemical modifications thereof. Modifications of the nucleosides include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at cytosine exocyclic amines, substitution of 5-bromo-uracil, and the like.

"**Nucleotide**" as used herein is defined as a modified or naturally occurring deoxyribonucleotide or ribonucleotide. Nucleotides typically include purincs and pyrimidines, which include thymidine, cytidine, guanosine, adenine and uridine.

"**Oligonucleotide**" refers to a polynucleotide formed from a plurality of linked nucleotide units as defined above. The nucleotide units each include a nucleoside unit linked together, typically via a phosphate linking group. The term oligonucleotide also refers to a plurality of nucleotides that are linked together via linkages other than phosphate linkages. The oligonucleotide may be naturally occurring or non-naturally occurring. In a preferred embodiment the oligonucleotides of this invention have between 1-1,000 nucleotides.

"**Nucleic acid ligand**" as used herein is a nucleic acid having a desirable action on a target. A desirable action includes, but is not limited to, binding of the target, catalytically changing the target, reacting with the target in a way which modifies/alters the target or the functional activity of the target, covalently attaching to the target as in a suicide inhibitor, facilitating a reaction between the target and

another molecule. In the preferred embodiment, the action is specific binding affinity for a target molecule, such target molecule being a three dimensional chemical structure other than a polynucleotide that binds to the nucleic acid ligand through a mechanism which predominantly depends on Watson/Crick base pairing or triple helix binding, wherein the nucleic acid ligand is not a nucleic acid having the known physiological function of being bound by the target molecule. In one embodiment, the nucleic acid ligand is a non-naturally occurring nucleic acid. In preferred embodiments of the invention, the nucleic acid ligands are identified by the SELEX methodology. Nucleic acid ligands includes nucleic acids that are identified from a candidate mixture of nucleic acids, said nucleic acid ligand being a ligand of a given target, by the method comprising a) contacting the candidate mixture with the target, wherein nucleic acids having an increased affinity to the target relative to the candidate mixture may be partitioned from the remainder of the candidate mixture; b) partitioning the increased affinity nucleic acids from the remainder of the candidate mixture; and c) amplifying the increased affinity nucleic acids to yield a ligand-enriched mixture of nucleic acids.

"**Nucleic acid**" means either DNA, RNA, single-stranded or double-stranded and any chemical modifications thereof. Modifications include, but are not limited to, those which provide other chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and fluxionality to the nucleic acid ligand bases or the nucleic acid ligand as a whole. Such modifications include, but are not limited to, 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at exocyclic amines, substitution of 4-thiouridine, substitution of 5-bromo or 5-iodo-uracil, backbone modifications, methylations, unusual base-pairing combinations such as the isobases isocytidine and isoguanidine and the like. Modifications can also include 3' and 5' modifications such as capping.

"**DNA template**" refers to a deoxyribonucleotide which provides instructions for an RNA polymerase to assemble a complementary ribonucleotide copy in a process termed "transcription." The strand of DNA copied is called the "sense strand." The DNA template strand also provides signals to initiate the copy synthesis

by the enzyme at specific locations before the start of the sense strand and to terminate the copy synthesis at specific locations shortly after the end of the sense strand. The DNA template may be single-stranded or double-stranded. In a preferred embodiment, the DNA template is double-stranded.

"**Non-immunogenic, high molecular weight compound**" is a compound of approximately 1000 Da or more that typically does not generate an immunogenic response. An immunogenic response is one that induces the organism to produce antibody proteins. Examples of non-immunogenic, high molecular weight compounds include polyethylene glycol (PEG); polysaccharides, such as dextran; polypeptides, such as albumin; and magnetic structures, such as magnetite.

As used herein a "**macromolecule**" refers to a large organic molecule. Examples of macromolecules include, but are not limited to nucleic acids, oligonucleotides, proteins, peptides, carbohydrates, polysaccharides, glycoproteins, lipophilic compounds, such as cholesterol, phospholipids, diacyl glycerols and dialkyl glycerols, hormones, drugs, non-immunogenic high molecular weight compounds, fluorescent, chemiluminescent and bioluminescent marker compounds, antibodies and biotin, etc without limitation.

"**Bioconjugate**" as defined herein refers to any oligonucleotide which has been derivatized with another molecular entity. In a preferred embodiment the oligonucleotide is derivatized with a macromolecule.

"**Bioconjugation**" or "**Conjugation**" refers to the derivatization of an oligonucleotide with another molecular entity. The "**molecular entity**" can be any molecule and can include a small molecule or another macromolecule. Examples of molecular entities include but are not limited to other macromolecules, polymers or resins, such as polyethylene glycol (PEG) or polystyrene, diagnostic detector molecules, such as biotin, fluorescein or coumarin, reporter enzymes, photoaffinity labels, steroids, pharmacokinetic modulators such as PEG, lipids or liposomes, reactive moieties for post-transcriptional conjugation such as a hexylamine or a diene or dienophile, and chelates for binding metals or any other modifying group. The terms bioconjugation and conjugation are used interchangeably throughout the Specification.

"**Therapeutic Agent**" means a compound which is used in the treatment or prevention of diseases and disorders.

"**Diagnostic Agent**" means a bioconjugate which can be used for detecting the presence or absence of and/or measuring the amount of a target in a sample. Detection of the target molecule is mediated by its binding to a nucleic acid component of a bioconjugate specific for that target molecule. The bioconjugate can be labeled, for example radiolabeled, to allow qualitative or quantitative detection.

"**Improved pharmacokinetic properties**" means that a bioconjugate shows a longer circulation half-life *in vivo* relative to a nucleic acid that is not part of a bioconjugate, or has other pharmacokinetic benefits such as improved target to non-target concentration ratio.

"**Target**" refers to any compound upon which a nucleic acid can act in a predetermined desirable manner. A SELEX target molecule can be a protein, peptide, nucleic acid, carbohydrate, lipid, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, virus, pathogen, toxic substance, substrate, metabolite, transition state analog, cofactor, inhibitor, drug, dye, nutrient, growth factor, cell, tissue, etc., without limitation. Virtually any chemical or biological effector would be a suitable SELEX target. Molecules of any size can serve as SELEX targets. A target can also be modified in certain ways to enhance the likelihood of an interaction between the target and the nucleic acid.

Transcription from synthetic DNA templates using T7 RNA polymerase and the GAP molecules is a convenient and highly efficient method for synthesis of RNA derivatized exclusively at the 5'-position. In RNA polymerase catalyzed DNA template-dependent transcription, the enzyme uses one strand of DNA as a template to assemble a complementary RNA copy. The transcription of DNA by T7 RNA polymerase begins at a uniquely defined base relative to the promoter DNA sequence. No primer piece of RNA is required to start the copy synthesis. Successive nucleotide triphosphates are condensed such that the growth of the RNA copy is from the 5'-end to the 3'-end. The enzyme positions the first nucleotide (usually GTP or ATP) and the 3'-hydroxyl group of this nucleotide then reacts with the 5'-triphosphate of the incoming nucleoside. The 3'-hydroxyl group of the dinucleotide then

condenses with the next nucleotide brought into position; and so on. The synthesis is driven forward by the hydrolysis of pyrophosphate. Finally, and central to this invention, it has been found that the 5'-triphosphate of the first nucleotide in the nascent RNA is not involved in the transcription. The inventors have exploited this finding to develop a novel method for rapidly and conveniently synthesizing oligonucleotide bioconjugates.

The present invention provides a method for the enzymatic synthesis of bioconjugates comprising RNA derivatized exclusively at the 5'-position with a molecular entity. In its most basic form the method of the instant invention can be described by the following steps:

a) providing a DNA template and b) combining the DNA template with nucleotide triphosphates, a 5'-substituted guanosine and an RNA polymerase under conditions suitable for transcription. The types of nucleotide triphosphates used will depend on the composition of the template and the desired RNA product.

Using the method of this invention the 5'-modified guanosine can only be added at the initiating 5'-end of the transcript during the initiation phase of transcription. As stated above, transcript elongation is driven forward by the hydrolysis of pyrophosphate, therefore it is necessary that the remaining nucleotides be nucleoside triphosphates. The 5'-substituted guanosine does not have a 5'-triphosphate group and as such it can participate in initiation, but not elongation. Therefore, in contrast to other methods of enzymatically incorporating substituted nucleotide triphosphates during RNA synthesis, wherein substituted nucleotide triphosphates are incorporated throughout the RNA transcript, the method of the present invention provides a unique method of synthesizing bioconjugates comprising a molecular entity attached exclusively to the 5'-position of an oligonucleotide.

Since the first nucleotide in the DNA template to be transcribed is a cytosine, a 5'-derivatized guanosine (referred to herein as GAP) will compete with a GTP as the first component of the nascent RNA transcript. Thus, a mixture of RNA oligonucleotides containing bioconjugates comprising 5'-substituted RNA oligonucleotides and 5'-unsubstituted RNA oligonucleotides will be obtained. By

increasing the concentration of the 5'-derivatized guanosine in the transcription reaction relative to the GTP concentration, however, proportionally more derivatized guanosine will be incorporated into the transcript. As shown in Figure 1, a ratio of GAP:GTP of 10:1 results in 92 % of the transcript being initiated with GAP. Theoretically, if GAP and GTP are used as initiating nucleotides with equal efficiency, GAP should be present 90.91 % of the time. Depending on the required level of purity of the 5'-substituted transcript, the GAP-conjugate:GTP ratio can be varied.

The ability to use a 5'-derivatized guanosine as a substrate in the enzymatic synthesis of an oligonucleotide bioconjugate offers significant advantages over currently available methods or synthesizing these compounds. First, this method offers the ability to specifically incorporate a macromolecule at the 5'-position of the RNA oligonucleotide during enzymatic synthesis of the RNA oligonucleotide. This is in contrast to traditional and time-consuming methods of synthesizing bioconjugates, in which the oligonucleotide must be first chemically synthesized in a manner which incorporates a modified nucleoside during the last step of the synthesis, and then conjugating the modified oligonucleotide to a molecular entity. Second, the use of 5'-derviatized guanosines allows for the modification of transcripts which are too long to be chemically synthesized, greatly increasing the possible applications. Third, current methods known in the art for enzymatically incorporating modified nucleotide triphosphates into an nascent oligonucleotide result in the synthesis of modified nucleotides having several substituted nucleotides within the oligonucleotide. In contrast, the method of the present invention allows for the controlled, enzymatic synthesis of a bioconjugate substituted exclusively at the 5'-end of the oligonucleotide.

One embodiment of the present invention includes a method for generating high affinity bioconjugates to specific target molecules. In the preferred method, the nucleic acid ligand is identified by the SELEX method. The SELEX method is described in United States Patent Application Serial No. 07/536,428, filed June 11, 1990, entitled "Systematic Evolution of Ligands by EXponential Enrichment," now abandoned; United States Patent Application Serial No. 07/714,131, filed June 10,

1991, entitled "Nucleic Acid Ligands," now United States Patent No. 5,475,096; United States Patent Application Serial No. 07/931,473, filed August 17, 1992, entitled "Methods of Identifying Nucleic Acid Ligands," now United States Patent No. 5,270,163 (See also PCT Application Publication No. WO 91/19813). These applications, each specifically incorporated herein by reference, are collectively called the SELEX Patent Applications.

In its most basic form, the SELEX process may be defined by the following series of steps:

1) A candidate mixture of nucleic acids of differing sequence is prepared. The candidate mixture generally includes regions of fixed sequences (i.e., each of the members of the candidate mixture contains the same sequences in the same location) and regions of randomized sequences. The fixed sequence regions are selected either: (a) to assist in the amplification steps described below, (b) to mimic a sequence known to bind to the target, or (c) to enhance the concentration of a given structural arrangement of the nucleic acids in the candidate mixture. The randomized sequences can be totally randomized (i.e., the probability of finding a base at any position being one in four) or only partially randomized (e.g., the probability of finding a base at any location can be selected at any level between 0 and 100 percent).

2) The candidate mixture is contacted with the selected target under conditions favorable for binding between the target and members of the candidate mixture. Under these circumstances, the interaction between the target and the nucleic acids of the candidate mixture can be considered as forming nucleic acid-target pairs between the target and those nucleic acids having the strongest affinity for the target.

3) The nucleic acids with the highest affinity for the target are partitioned from those nucleic acids with a lesser affinity to the target. Because only an extremely small number of sequences (and possibly only one molecule of nucleic acid) corresponding to the highest affinity nucleic acids exist in the candidate mixture, it is generally desirable to set the partitioning criteria so that a significant amount of the nucleic acids in the candidate mixture (approximately 5-50%) are retained during partitioning.

4) Those nucleic acids selected during partitioning as having the relatively higher affinity to the target are then amplified to create a new candidate mixture that is enriched in nucleic acids having a relatively higher affinity for the target.

5) By repeating the partitioning and amplifying steps above, the newly formed candidate mixture contains fewer and fewer unique sequences, and the average degree of affinity of the nucleic acids to the target will generally increase. Taken to its extreme, the SELEX process will yield a candidate mixture containing one or a small number of unique nucleic acids representing those nucleic acids from the original candidate mixture having the highest affinity to the target molecule.

The SELEX Patent Applications describe and elaborate on this process in great detail. Included are targets that can be used in the process; methods for partitioning nucleic acids within a candidate mixture; and methods for amplifying partitioned nucleic acids to generate enriched candidate mixtures. The SELEX Patent Applications also describe ligands solutions obtained to a number of target species, including both protein targets where the protein is and is not a nucleic acid binding protein. The SELEX Patent Applications describe a number of uses for nucleic acid ligands including numerous therapeutic and diagnostic uses.

In this embodiment the bioconjugate is prepared by the SELEX method as described in the SELEX Patent Applications. Briefly, bioconjugates to a target are identified by the SELEX method by the steps comprising:

1) preparing a candidate mixture of bioconjugates by the steps comprising (a) providing a DNA template having a sequence to be transcribed and (b) combining the DNA template with nucleotide triphosphates, a modified guanosine, and an RNA polymerase under conditions suitable for transcription;

2) contacting the bioconjugate candidate mixture with a target, wherein bioconjugates having an increased affinity to the target relative to the bioconjugate candidate mixture may be partitioned from the remainder of the bioconjugate candidate mixture;

3) partitioning the increased affinity bioconjugates from the remainder of the bioconjugate candidate mixture; and

4) amplifying the increased affinity bioconjugates to yield a ligand-enriched mixture of bioconjugates, whereby bioconjugates of the target are identified. The 5'-substituted GAP can aid in (1) the SELEX partition step, e.g. BIA-SELEX (see United States Application Serial No. 08/792,075, filed January 31, 1997, entitled "Flow Cell SELEX, which is incorporated herein by reference), plate SELEX (Conrad *et al.* (1996) Methods of Enzymol. 267:336), and streptavidin column partitions, (2) monitoring the progress of a SELEX using a reporter substitution, and/or (3) participating directly with the target protein, e.g., Blended SELEX (United States Patent No. 5,683,867, issued November 04, 1997, entitled "Systematic Evolution of Ligands by Exponential Enrichment: Blended SELEX").

Using this method, nucleic acid ligands derivatized exclusively at the 5'-position of the nucleic acid ligand with virtually any molecular entity which is compatible with transcription can be prepared and identified. Molecular entities that can be coupled to nucleic acid ligands include, but are not limited to lipophilic molecules, proteins, peptides, reporter molecules, reporter enzymes and steroids.

In another embodiment of this invention the 5'-derivatized guanosine contains a reactive moiety which can be used for post-transcription conjugation of the transcript. This embodiment of the invention can be described by the following steps: a) providing a DNA b) combining the DNA template with nucleotide triphosphates, a 5'-substituted guanosine, wherein said 5'-substituent contains a reactive moiety and an RNA polymerase under conditions suitable for transcription; and c) reacting the product from step b) with a molecular entity containing a moiety capable of reacting with the reactive moiety on said 5'-substituent. Post transcription conjugation is necessary to obtain oligonucleotides derivatized with molecular entities which are not compatible with transcription. Examples of reactive moieties include but are not limited to amines, dienes, dienophiles, thiols, vinylsulfones, photoaffinity labels and interchelators.

In certain embodiments, the molecular entity may provide certain desirable characteristics to the nucleic acid ligand, such as, increasing RNA hydrophobicity and enhancing binding, membrane partitioning and/or permeability. Additionally, reporter molecules, such as biotin, fluorescein, or peptidyl metal chelates for

incorporation of diagnostic radionuclides may be added, thus providing a bioconjugate which may be used as a diagnostic agent.

Example 1 describes the synthesis of a variety of 5'-modified guanosine monophosphates. For commonly used functional groups it is more efficient to conjugate the moiety of interest to the GAP molecule prior to transcription. This allows for large scale synthesis of the initiator, pre-transcription purification of the initiator, and negates the need for post-transcriptional conjugations. The modified guanosines synthesized are set forth in Schemes 1, 2, 4 and 5 and include GAP **(5)**, GAP-fluorescein **(11)**, GAP-biotin **(12)**, GAP-Tc chelate **(13)**, GAP-TEG **(10)**, GAP-TEG-fluorescein **(14)**, GAP-TEG-biotin **(15)** and GAP-TEG-Tc chelate **(16)**. Biotin and fluorescein are very common conjugates which provide very useful properties for RNAs. The GAP-TEG analogs were synthesized because they are less expensive, less hydrophilic and potentially less immunogenic than the GAP analogs. When conjugating GAP to more hydrophobic adducts, such as biotin, solubility in aqueous buffers becomes a serious consideration.

Example 2 illustrates the feasibility of using transcription with 5'-modified guanosines to synthesize oligonucleotides modified exclusively at the 5'-position. This example demonstrates that both GAP and GAP conjugates can compete with GTP for the initiation of RNA synthesis. As shown in **Figure 1**, a ratio of GAP:GTP of 10:1 results in 92 % of the transcript being initiated with GAP. Example 2 also illustrates that the yield of full length product does not decrease as a result of GAP-Biotin inhibiting the transcription reaction.

Example 3 illustrates the post transcription conjugation of a GAP initiated transcript. Post transcription conjugation is necessary to obtain oligonucleotides derivatized with molecular entities that are not compatible with transcription. Transcription with primary amine initiators allows for the post-transcriptional conjugation of RNA with a wide variety functional groups through easily available NHS chemistry. In this example a GAP initiated RNA was reacted with a biotin NHS ester.

Example 4 demonstrates that GAP **(5)** and GAP-TEG **(10)** incorporate to the same extent resulting in the same amount of full length 5'-modified oligonucleotide product.

Example 5 (Figure 4A) demonstrates that GAP conjugate compounds **11-16** incorporate to the same extent resulting in the same amount of full length product as GTP (see Table below). This example compares the transcription reactions run with GAP analogs **11-16** and a control run without GAP. The results are set forth in the table below. Figure 4B shows that the full length transcripts initiated with GAP-fluorescein **(11)** and GAP-TEG-fluorescein **(14)** result in a fluorescent signal upon irradiation with ultra violet light. This example clearly demonstrates that virtually any linker or conjugate attached to guanosine, which ultimately is compatible with the transcription enzymes, could be used to enzymatically derivatize the 5'- terminal end of an RNA molecule.

Lanes (Figure 4A)	**pmoles eluted**
1,2 transcript without GAP	1572
3 GAP-TEG	1948
4 GAP-Biotin	1810
5 GAP-TEG-Biotin	2369
6 GAP-Tc chelate	2032
7 GAP-TEG-Tc chelate	1984
8 GAP-Fluorescein	2426
9 GAP-TEG-Fluorescein	2614

Example 6 describes the labeling of a GAP-Tc chelate **(13)** initiated transcript with ^{99}m Tc.

The following examples are presented for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLES

General methods. All reagents and solvents were used as received from the manufacturer. 5'-dimethoxytrityl-^2N-isobutyrylguanosine **(1)** was purchased from ChemGenes Corp. The 5' Amino-Modifier C6-TFA was purchased from Glen Research. All reactions were carried out under anhydrous conditions with inert atmosphere in oven-dried glassware. TLC was performed on Baker Si250F TLC Plate-Silica Gel and the spots were rendered visible by UV light. Flash chromatography was performed with a Biotage Flash 40 apparatus using a Kiloprep Column (KP-Sil, 60 A). RP-HPLC was performed on a Waters' Delta Pak 5 μ C18 300 A, 3.9 x 150 mm column. Buffer A: 100 mM TEAA at pH 7.0; Buffer B: ACN. The temperature was 30°C and the flow rate was 0.50 mL/min. NMR spectra were recorded on a Bruker ARX 300 spectrometer using $CDCl_3$ and $(CD_3)_2SO$ as solvents with TMS as an internal standard. Electrospray mass spectrometry was performed on a Fissions Quattro II (Beverly, MA) using negative ion mode. The samples were delivered in a 1:1 MeOH/H_2O (v/v) containing 0.1% TEA at 10 μL/min to the mass spectrometer.

General *in vitro* transcription. T7 RNA polymerase was purchased from Enzyco, Denver, Colorado. 2'-F-CTP and -UTP were purchased from USB Biochemicals. The transcriptional template was a 104-bp DNA amplified by PCR from a linearized pUC plasmid with the sequence:

TAA TAC GAC TCA CTA TAG GGA GAC AAG AAT AAA CGC TCA AGC GGG ATT TTC CTG ATC ATC CCA CTG ATT CGG GGC CTT ACT TCG ACA GGA GGC TCA CAA CAG GC (SEQ ID NO:1)

where the bold bases represent the T7 promoter sequence and the underlined bases represent the PCR primer regions. *In vitro* transcription was performed under standard conditions (Krupp and Soll (1987) FEBS 212:271-275; Milligan and Uhlenbeck (1989) Methods of Enzymology 180:51-62), which were modified for the incorporation of 2'-F pyrimidine triphosphates into the transcript (Lin *et al.* (1994) Nucleic Acids Res. 22:5229-34). Briefly, transcription was performed in 40 mM Tris-HCl, pH 8, 4% PEG 8000, 12 mM $MgCl_2$, 5 mM DTT, 1mM spermadine, 0.002 % Triton X-100 with a template concentration of 0.5 to 1.0 μM, and a T7 RNA

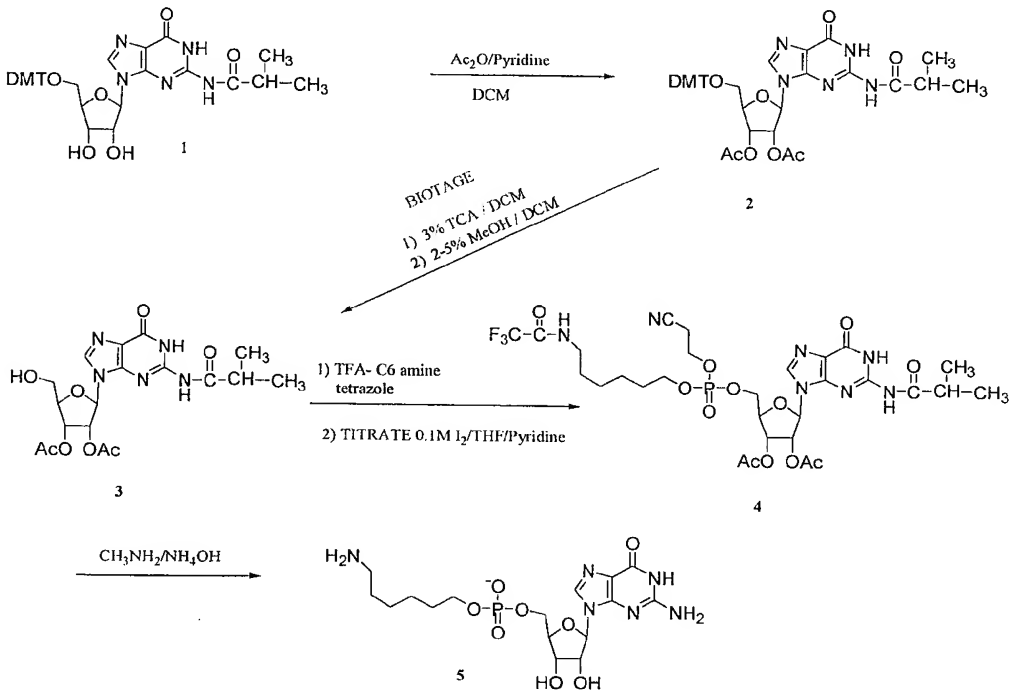
polymerase concentration of 0.4 μM. 2'-F-CTP and 2'-F-UTP were added to 3mM while ATP and GTP were added to a final concentration of 1 mM. Reactions were incubated at 37°C for 16-20 hours.

Example 1. Synthesis 5' substituted guanosine monophosphates

Small-scale solid phase synthesis of 5'-(O'-hexylamino)guanosine monophosphate (GAP). Initially, GAP **(5)** was synthesized with commercially available reagents using a DNA/RNA synthesizer. Starting with acetate protected guanosine CPG (Glen Research) a single coupling step was used to add 5'-Amino-modifier C6 phosphoramidite (Glen Research). The product was cleaved from the solid support, deprotected with NaOH and purified by reverse phase chromatography to yield GAP **(5)**. The success of this experiment stimulated the larger scale production of the GAP molecule, discussed below.

Large scale solution phase synthesis of 5'-(O'-hexylamino)guanosine monophosphate **(5)**. Scheme 1 sets forth the large scale solution phase synthesis of GAP **(5)**.

SCHEME 1



2',3'-diacetyl-5'-dimethoxytrityl-^2N-isobutyrylguanosine (2). The 5'-dimethoxytrityl-^2N-isobutyrylguanosine **(1)** (5 g, 7.63 mmol) was dissolved in 30 mL of pyridine and 15 mL of dichloromethane (DCM) at 23 °C and 3.6 mL (38 mmol) of acetic anhydride was added. The reaction was complete in (5 hours) as determined by TLC (9.5:9.5:1 hexane/EtOAc/MeOH). The solution was then brought up in EtOAc (500 mL) and washed with saturated $NaHCO_3$ (3 x 300 mL). The $NaHCO_3$ fractions were back extracted with EtOAc. The EtOAc fractions were combined, dried over $MgSO_4$ and concentrated to a dry precipitate *in vacuo*. This afforded 5.5 g (97.6%) of pure product **2** as determined by ^{1}H NMR (300 MHz, $(CD_3)_2SO$).

2',3'-diacetyl-^2N-isobutyrylguanosine (3). The 2',3'-diacetyl-5'-dimethoxytrityl-^2N-isobutyrylguanosine **(2)** (5.5 g, 7.44 mmol) was brought up in 10 mL of DCM and loaded onto a Biotage Flash 40 silica gel column. The dimethoxytrityl was removed on the column using a of 3 % solution of trichloroacetic acid (TCA) and 0.5 % MeOH in DCM.. After the dimethoxytrityl had eluted from the column, as determined visually and by TLC (19:1 DCM/MeOH), the column was washed with 10 volumes of 0.5% MeOH in DCM. The product was then eluted with an increasing gradient of 1 %-10 % MeOH in DCM. The appropriate fractions were combined and concentrated to a solid *in vacuo.* This afforded 2.96 g (91 %) of pure product **3** as determined by ^{1}H NMR (300 MHz, $(CD_3)_2SO$).

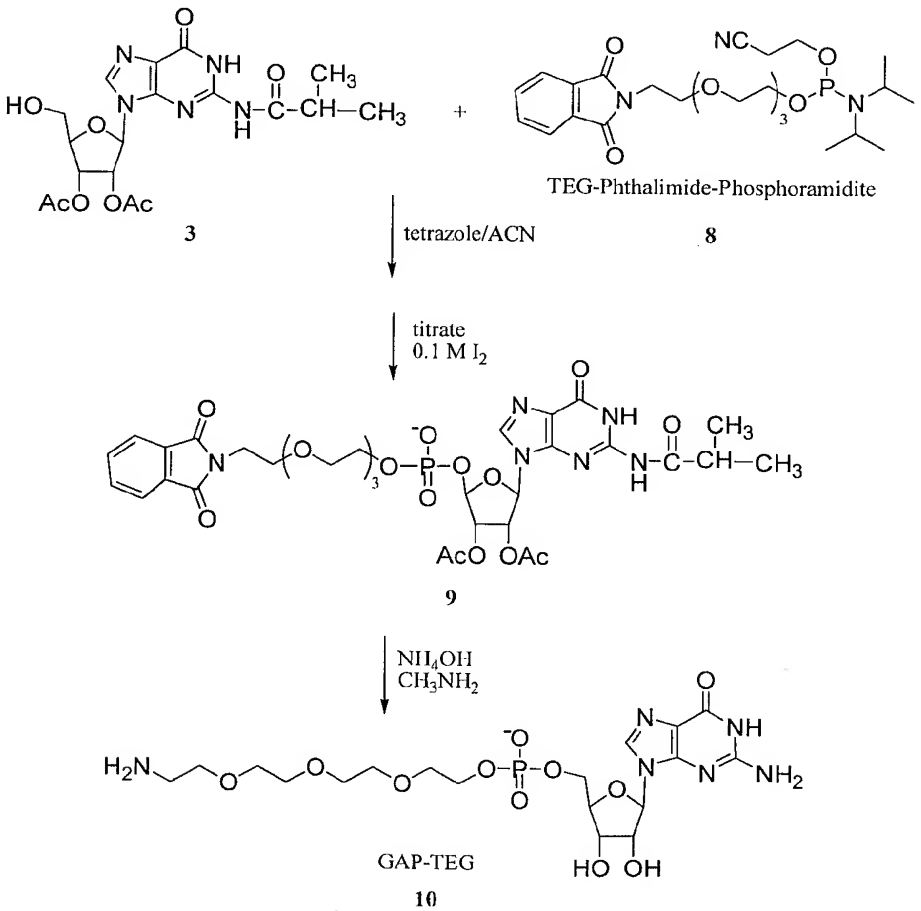
5'-(O'-hexylamino)guanosine monophosphate (GAP) (5). To a stirred solution of **3** (2.96 g, 6.76 mmol) in dry ACN (20 mL) was added 5' Amino-Modifier C6-TFA (3.64 g, 1.3 equiv; Glen Research) and 0.47 M tetrazole in anhydrous ACN (374 mL, 26 equiv; Glen Research). The reaction was complete in four hours as determined by TLC. The solution was titrated with 0.1 M oxidizing solution (I_2/THF/pyridine) (100 mL; Glen Research) until a brown color persisted. The solution was concentrated *in vacuo* to approximately one fifth of its volume and then brought up in EtOAc (500 mL) and washed with 5 % $NaHSO_3$ (2 x 300 mL) and saturated $NaHCO_3$ (2 x 300 mL). The aqueous washes were back extracted with EtOAc (500 mL) and the EtOAc fractions were combined, dried over $MgSO_4$ and then concentrated to dryness

in vacuo. This afforded a crude yield of 5.57 g (109.8%) of **4**. This crude material was then treated with 1:1 NH_4OH/CH_3NH_2 (125 equiv:125 equiv) for 30 minutes at 65°C. The NH_4OH and CH_3NH_2 were removed *in vacuo* and the remaining material was purified via RP-HPLC to obtain 2.5 g (80%) of **5** as a solid. Product was verified by ^1H NMR (300 MHz, $(CD_3)_2SO$), and MS calculated for $C_{16}H_{26}N_6O_8P$ (M+1): 462.3.

Synthesis of 5'-(O'-tetraethylene glycol)guanosine monophosphate (GAP-TEG) **(10)**.

5'-(O'-tetraethylene glycol)guanosine monophosphate (GAP-TEG) **(10)** was synthesized by reaction of compound **3** with TEG-Phthalimide phosphoramidite **(8)** as outlined in Scheme 2. The synthesis of TEG-Phthalimide phosphoramidite is outlined in Scheme 3 below.

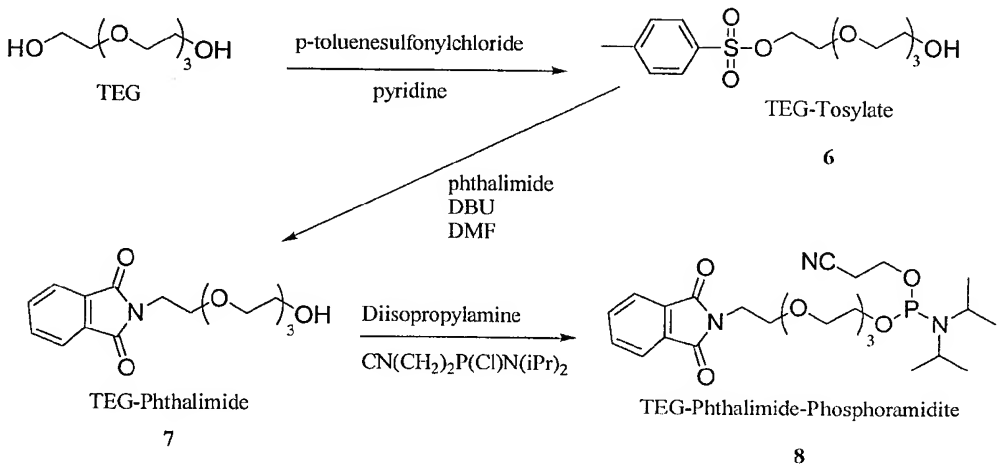
SCHEME 2



5'-(O'-tetraethylene glycol)guanosine monophosphate (GAP-TEG) **(10)**. To a stirred solution of **3** (0.941 g, 2.15 mmol) in dry ACN (20 mL) was added 0.47 M tetrazole in anhydrous ACN (120 mL, 56.4 mmol; Glen Research) and 1.89 g of crude **8**. The reaction was complete in four hours as determined by TLC. The solution was titrated with 0.1 M oxidizing solution (32 mL; Glen Research) until a brown color persisted. The solution was concentrated *in vacuo* to approximately one fifth of its volume, taken up in EtOAc (500 mL) and washed with 5 % $NaHSO_3$ (2 x 300 mL) and saturated $NaHCO_3$ (2 x 300 mL). The aqueous washes were back extracted with EtOAc (500 mL). The organic fractions were combined, dried over $MgSO_4$ and concentrated to dryness *in vacuo*. This afforded a crude yield of 2.2 g (118.8%) of compound **9**. The crude material was treated with 1:1 NH_4OH/CH_3NH_2 (125 equiv: 125 equiv) for 30 minutes at 65 °C. The NH_4OH and CH_3NH_2 were removed *in vacuo* and the remaining material was purified via RP-HPLC to obtain 0.900 g (77.95%) of pure product compound **10** as determined by ^1H NMR (300 MHz, $(CD_3)_2SO$) and MS calculated for $C_{18}H_{29}N_6O_{11}P$ (M+1): 537.1.

Tetraethylene glycol phthalimide phosphoramidite **(8)** was synthesized as outlined in Scheme 3.

SCHEME 3



Tetraethylene glycol monotosylate **(6)**. Tetraethylene glycol (100 mL, 575 mmol) was dissolved in 250 mL of pyridine and cooled to 0 °C and treated with 11.0 g (0.1

eq., 57.5 mmol) *p*-toluenesulfonyl chloride. When solution was complete, the reaction was stored in the refrigerator overnight. The reaction was complete as determined by TLC (19:1 EtOAc/MeOH). The reaction mixture was then concentrated *in vacuo*. The residue was dissolved in 600 mL of EtOAc and extracted with H_2O (3 x 200 mL). The H_2O fractions were back-extracted with 400 mL of EtOAc and the combined EtOAc fractions were extracted with saturated aqueous Na_2HPO_4. The organic phase was dried over $MgSO_4$ and concentrated *in vacuo* to yield 18.0 g (90.2 % crude) of tetraethylene glycol monotosylate **(6)** as a colorless oil. ^1H NMR (300 MHz, $CDCl_3$) δ 7.77 (d, J = 8.1 Hz, 2H), 4.13 (t, J = 4.8 Hz, 2H), 3.68-3.53 (m, 14 Hz), 2.58 (t, J = 5.6 Hz, 1H), 2.42 (s, 3H).

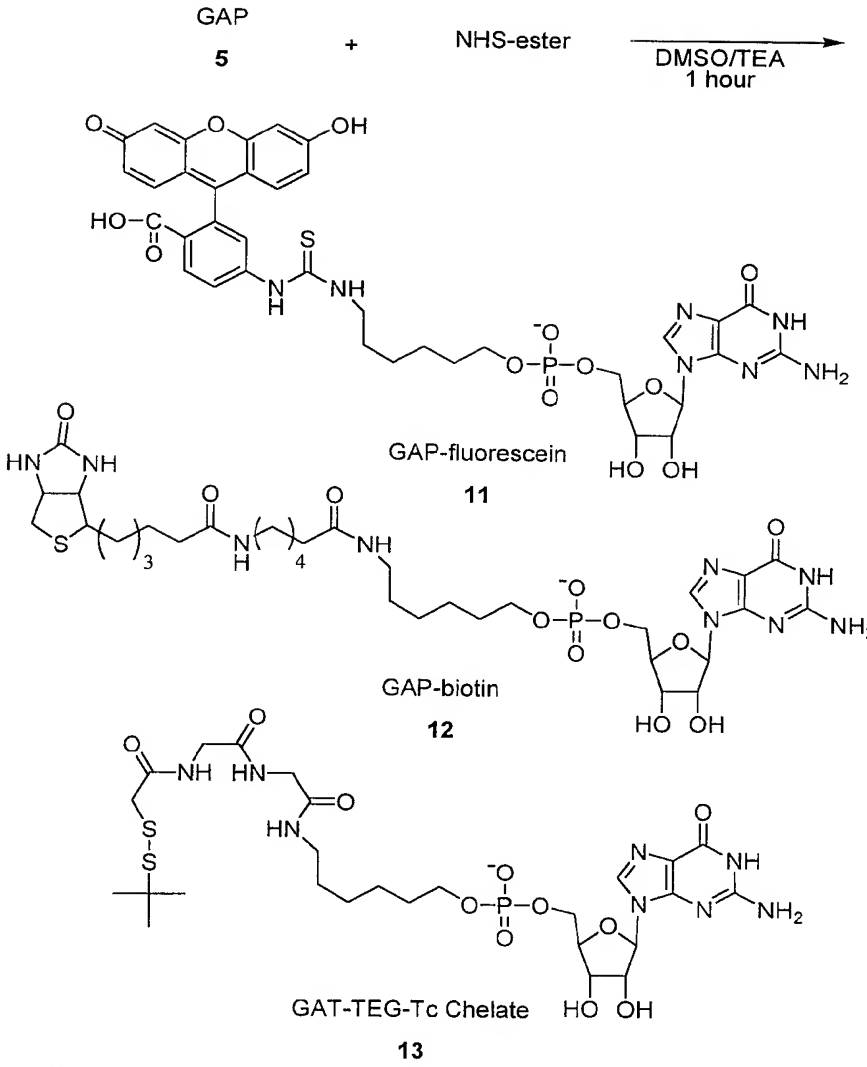
Tetraethylene glycol monophthalimide **(7)**. To a stirred solution of 18.0 g (51.7 mmol) of crude **6** in 225 mL of anhydrous DMF was added 8.0 g (1.05 eq., 54.3 mmol) of phthalimide and 8.12 mL (1.05 eq., 54.3 mmol) of 1,8-diazabicyclo[5.5.0]undec-7-ene. The solution was heated at 70°C for 18 hours and then concentrated *in vacuo*. The reaction was determined complete by TLC (19:1 EtOAc/MeOH). The crude yellow oil was purified by flash chromatography using a Biotage Flash 40 silica gel column and eluting with 25% EtOAc/hexane, 50% EtOAc/hexane, 75% EtOAc/hexane, EtOAc, and then 10% MeOH to afford 13.7 g (82%) of compound **7** as an oil. Upon standing, **7** became a waxy white solid. ^1H NMR (300 MHz, $CDCl_3$) δ 7.84-7.78 (m, 2H), 7.70-7.66 (m, 2H), 3.86 (t, J = 5.6 Hz, 2H), 3.70 (t, J = 5.6 Hz, 2H), 3.64-3.51 (m, 12H), 2.67 (bs, 1H).

1-Phthalimido-tetraethylene glycol(diisopropylamino)-β-cyanoethoxyphosphine **(8)**. A 1 g (3.1 mmol) aliquot of **7** was brought up in of THF (20 mL), dried *in vacuo* to an oil and then resuspended in THF (20 mL) under argon. To this stirred solution was added *N,N*-diisopropylethylamine (702 µL, 4.0 mmol) and 2-cyanoethyl diisopropylchlorophosphoramidite (762 µL, 3.41 mmol; Aldrich). The reaction was complete in 30 minutes as determined by TLC. The solution was dried *in vacuo* to an oil, taken up in EtOAc (300 mL), washed with H_2O (2 x 100 mL) and the organic

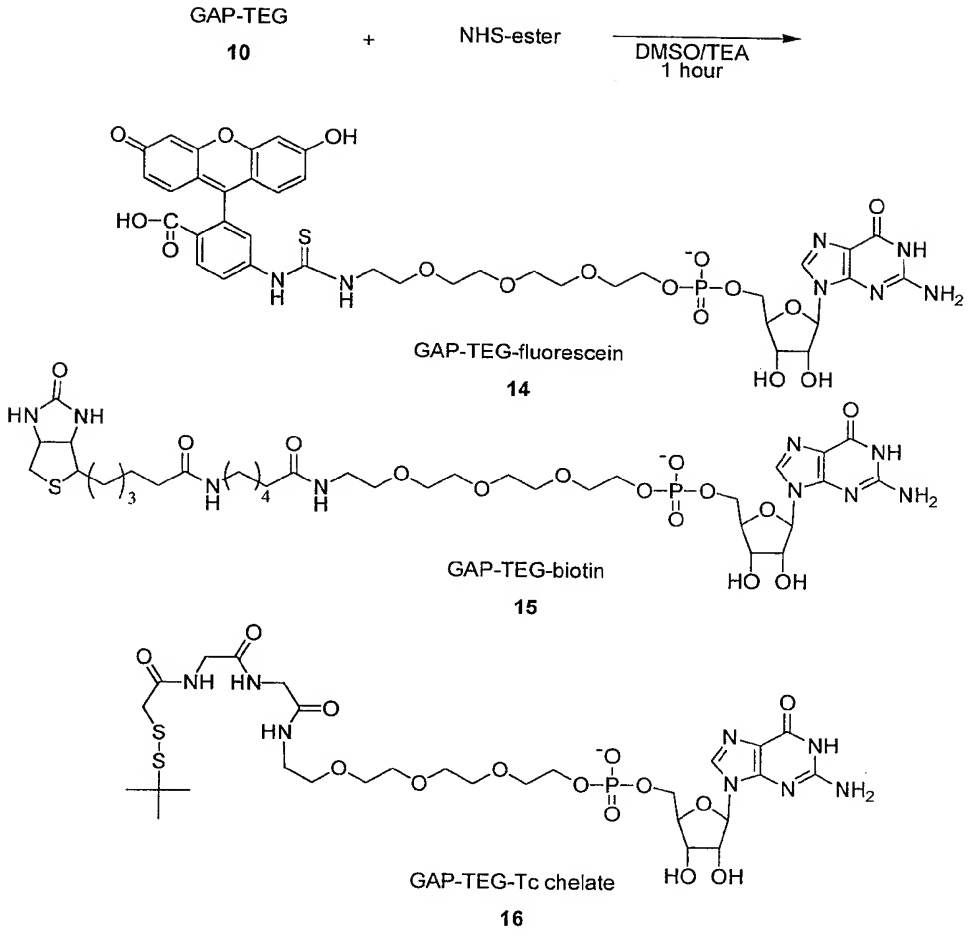
phase was dried over $MgSO_4$. This afforded a crude yield of 1.89 g (116.5%) of compound **8** which was used without further purification.

General method for conjugation of NHS ester activated molecules to the 5'-amino-terminus of compounds **5** (Scheme 4) and **10** (Scheme 5). To a solution of **5** or **8** (10 mmol) in DMSO (219.5 μL, 20 mg/mL) and TEA (11.5 μL, 5%) was added the desired NHS ester molecule (20 mmol). The reaction was complete in one hour as determined by RP-HPLC. Purification of the 5' modified guanosine monophosphates was achieved by reverse phase chromatography. Separation was achieved by loading 1 gram of crude reaction mixture on a Waters Delta Pak C-18 column (50 X 300 mm) column and running a 2% to 50% gradient (108 min) of acetonitrile in 100 mM triethylamine carbonate pH 8 at 12 mL/min. Analytical separations were determined with a Waters Delta Pak C-18 (4.9 X 150 mm) column and running 2% to 50% gradient (54 min) of acetonitrile in 100 mM triethylamine acetate pH 7 at 1 mL/min. Purification by RP-HPLC afforded yields of (18 mmol, 90%) of compounds **11-16** (Schemes 3 & 4).

SCHEME 4


GAP
5
+
NHS-ester
DMSO/TEA
1 hour
GAP-fluorescein
11
GAP-biotin
12
GAT-TEG-Tc Chelate
13

SCHEME 5



Biotinamidocaproate 5'-(O'-hexylamino)-guanosine monophosphate **(14)**.

To a solution of **5** (1 mmol) in DMSO (21.85 mL, 20 mg/mL) and TEA (1.15 mL, 5%) was added biotinamidocaproate *N*-hydroxysuccinimidyl ester (909 mg, 2 equiv). The reaction was complete in one hour as determined by RP-HPLC. Purification by RP-HPLC afforded a yield of 719.8 mg (90%) of pure product compound **14**.

Example 2. Initiation competition assays

GAP / γ-^{32}P-GTP initiation competition assay. A series of transcription reactions were performed using γ-^{32}P-GTP to determine the extent to which GAP **(5)** would compete with GTP for initiation of transcription reactions. The reactions were run under the standard conditions set forth above. The reactions were performed with the

GAP molecule added to a final concentration of 0 to 10 mM while GTP (1 mM) and gamma labeled ^{32}P-GTP were held at fixed concentrations. Since the ^{32}P is in the gamma position, only those GTP molecules which initiate transcription, will result in the incorporation of a radiolabel into the transcript. The reaction products were analyzed by denaturing gel electrophoresis. Full length transcript bands were excised from the gel, the RNA was eluted from the gel slices and was quantitated by UV absorbance at 260 nm. Percent incorporation was calculated with the following equation:

$$(1\text{-}CPM_{[GAP]}/CPM_{[init.]}) \text{ X } 100$$

where $CPM_{[GAP]}$ = the CPM of the full length band at a given GAP concentration and $CPM_{[init.]}$ = the CPM of the full length band at a GAP concentration of 0 mM. The results are depicted in Figure 1, which shows that an increase in concentration of GAP results in a corresponding decrease in γ-^{32}P GTP incorporation (**Figure 1**). Each reaction resulted in the same yield of full length transcript. Quantitation indicates that GAP is used as an initiating nucleoside to the same extent as GTP.

GAP-TEG-biotin / γ-^{32}P-GTP initiation competition assay. A second series of reactions were performed using a fixed amount of GTP (1 mM) and γ-^{32}P GTP and an increasing concentration of GAP-TEG-biotin **(15)** to give ratios of: 0, 0.1, 0.5, 1, 2, 4, and 10 to 1 GTP. The reaction products were analyzed by denaturing gel electrophoresis, which showed that the increase in GAP-TEG-biotin resulted in a corresponding decrease in γ-^{32}P GTP incorporation. (**Figure 2**). The competition by GAP-TEG-biotin is in agreement with the ratio of GAP-TEG-biotin to GTP.

GAP- Biotin inhibition assay. Transcription reactions were performed under the standard conditions set forth above, except that α-^{32}P-ATP was added to internally label the transcription products. The same series of transcription reactions with GTP to GAP-biotin **(12)** ratios again between 0 and 10 fold excess was used. As the amount of GAP in each reaction increased the amount of full length product remained the same, demonstrating that GAP-biotin does not inhibit RNA transcription.

Streptavidin Shift assay. Transcription reaction products for the 0 to10 ratios of GAP- biotin were combined with 10 µM streptavidin in 37.5 mM Tris, pH 7.5. The

products of the reaction were analyzed on a denaturing gel and quantified by phosphoimager. The amount of Streptavidin shift was correlated to the theoretical amount of GAP-biotin that should have been incorporated (**Figure 3**).

GAP-biotin incorporation

The presence of the 5' primary amine was assessed by the ability to conjugate NHS-biotin to the GAP transcript. Transcripts were synthesized in the presence of increasing concentrations of GAP. The resulting transcripts were conjugated with a 100-fold excess of NHS-biotin. After conjugation transcripts were admixed with a 5-fold excess of Streptavidin and applied to an 8% urea polyacrylamide gel. These data indicate that there is no biotin incorporation in the absence of GAP and that the percentage of gel-shifted transcript increases with increasing GAP added to the transcription reaction. The gel shift assay reaches a maximum at approximately 70 %, underestimating the GAP incorporation at the highest GAP:GTP ratios as compared to the γ-^{32}P-ATP-GTP assay or HPLC analysis of the conjugated transcript.

Repeating the same experiments with GAP-TEG instead of GAP will result in an identical outcome.

Example 3. Post transcription biotinylation of GAP RNA transcripts

A GAP initiated RNA was transcribed at a GAP to GTP concentration of 15:1. The 5' amine on the terminus of the unpurified full length GAP RNA (TEA salt) was then conjugated to a long chain biotin NHS ester in anhydrous DMSO /10% TEA. The biotin NHS ester was added at 5 eq. of biotin for one hour followed by an additional 5 eq. for one hour. The biotinylated transcript was purified by ethanol precipitation to remove free biotin. To verify post transcription conjugation the transcription reaction products were combined with a large excess of streptavidin (10 µM streptavidin in 37.5 mM Tris, pH 7.5). The products were analyzed on a denaturing gel to show a steptavidin shift. In the presence of streptavidin 50 percent of the biotin conjugated RNA material shifted to the slower migrating Streptavidin complex.

Example 4. Transcription with GAP **(5)** and GAP-TEG **(10)**

Transcription reactions were performed under standard conditions as set forth above, with GAP **(5)** or GAP-TEG **(10)** added at a ratio of 10 to 1 over the concentration of GTP. The reaction products were analyzed on a denaturing gel, which showed that transcription with GAP and GAP-TEG resulted in the same yield of full length transcript.

Example 5. Transcription with GAP and GAP-TEG conjugates

This example describes the incorporation of GAP conjugates **(11-13)** and GAP-TEG conjugates **(14-16)**. Transcription reactions were performed in parallel under standard conditions as set forth above using a 104-bp transcriptional template with the addition of α-^{32}P-ATP. The GAP conjugates were added at a ratio of 10 to 1 over the concentration of GTP. A control reaction was run in which no modified guanosine was added to the standard RNA transcription reaction. The reaction products were analyzed on an 8% polyacrylamide gel containing 7 M urea. The gel was visualized by autoradiography. (**Figure 4A**). The bands corresponding to full-length transcript were cut out of the gel, eluted and quantitated by UV spectroscopy. Analysis showed that all of the modified guanosine compounds resulted in the same yield of full length transcript as the control. Lanes 8 and 9 were transilluminated to detect the presence of fluorescein in the full length GAP-fluorescein and GAP-TEG-fluorescein transcripts. In all cases the 10 fold excess of the modified guanosine over GTP concentration showed no diminution of transcription yield.

Example 6. Labeling of GAP-Tc chelate **(13)** transcript

To 1 nmole GAP-Tc chelate **(13)** initiated transcript was added 200 µL of 100 mM $NaPO_4$ buffer, pH 8.5, 23 mg/mL NaTartrate, and 50 µL ^{99m}Tc pertechnetate (5.0 mCi) eluted from a 99-Mo column within 12 hours of use. The labeling reaction was initiated by the addition of 10 µL 5 mg/mL $SnCl_2$. The reaction mixture was incubated for 15 minutes at 90°C. The reaction was separated from unreacted ^{99m}Tc by spin dialysis through a 30,000 MW cut-off membrane (Centrex, Schleicher & Scheull) with two 300 µL washes. This labeling protocol results in 30-50% of the

added ^{99}m Tc being incorporated with a specific activity of 2-3 mCi/nmole RNA. ^{99}m Tc labeled transcript was analyzed by 8% polyacrylamide denaturing gel electrophoresis in the absence of EDTA. Quantitation indicates that over 97% of the ^{99}m Tc is associated with the full-length transcipt after spin dialysis (**Figure 5**).

CLAIMS

1. A method of synthesizing bioconjugates comprising:

(a) providing a DNA template for transcription, wherein the first nucleotide to be transcribe is a cytosine; and

(b) combining the DNA template with nucleotide triphosphates, a guanosine derivatized at the 5'-positition of the ribose ring with a molecular entity and RNA polymerase under conditions suitable for transcription.

2. The method of claim 1 wherein said molecular entity is selected from the group consisting of the 5'-labeled guanosine is selected from the group consisting of a macromolecule, polymer, resin, diagnostic detector molecule, reporter enzyme, photoaffinity label, steroid, pharmacokinetic modulator, reactive moietie for post-transcriptional conjugation and chelates for binding metals.

3. The method of claim 2 wherein said polymer is selected from the group consisting of PEG and polystyrene.

4. The method of claim 2 wherein said diagnostic detector molecule is selected from the group consisting of biotin, fluorescein and coumarin.

5. The method of claim 2 wherein said pharmacokinetic modulator is a liposome.

6. The method of claim 2 wherein said reactive moiety is selected from the group consisting of a hexylamine, diene or dienophile.

7. The method of claim 1 wherein said guanosine derivatized at the 5'-positition of the ribose ring is selected from the group consisting of GAP, GAP-fluorescein, GAP-biotin, GAP-Tc chelate, GAP-TEG, GAP-TEG-fluorescein, GAP-TEG-biotin and GAP-TEG-Tc chelate.

8. The method of claim 1 wherein the RNA polymerase is T7 RNA polymerase.

9. The method of claim 1 wherein said nucleotide triphosphates are modified at the 2'-position of the ribose ring.

10. The method of claim 1 wherein said nucleotide triphosphates are modified at the 5-position of the base.

11. The method of claim 1 wherein said molecular entity is selected for use as a therapeutic agent, diagnostic agent, or for use in radiotherapy.

12. The method of claim 1 wherein said molecular entity is selected to improve pharmokinetic behavior, to increase hydrophobicity, to enhance binding, to enhance membrane partitioning and to enhance permeability.

13. A product formed by the method of claim 1.

14. A method of identifying bioconjugates to a target comprising:

(a) preparing a candidate mixture of bioconjugates by the method comprising:

(i) providing a DNA template and

(ii) combining the DNA template with nucleotide triphosphates, a guanosine derivatized at the 5'-positition of the ribose ring with a molecular entity and RNA polymerase under conditions suitable for transcription;

(b) contacting the candidate mixture of bioconjugates with a target, wherein bioconjugates having increased affinity for the target may be partitioned from the remainder of the bioconjugate candidate mixture;

(c) partitioning the increased affinity bioconjugates from the remainder of the bioconjugate candidate mixture; and

(d) amplifying the increased affinity bioconjugates to yield a ligand-enriched mixture of bioconjugates, whereby bioconjugates to a target may be identified.

15. The method of claim 14 further comprising:

(e) repeating steps (b)-(d).

16. The method of claim 14 wherein said bioconjugate comprises an nucleic acid ligand derivatized with a molecular entity at the 5'-end of the nucleic acid ligand.

17. The method of claim 14 wherein said molecular entity is selected from the group consisting of a macromolecule, polymer, resin, diagnostic detector molecule, reporter enzyme, photoaffinity label, steroid, pharmacokinetic modulator, reactive moiety for post-transcriptional conjugation and chelates for binding metals.

18. The method of claim 17 wherein said polymer is selected from the group consisting of PEG and polystyrene.

19. The method of claim 17 wherein said diagnostic detector molecule is selected from the group consisting of biotin, fluorescein and coumarin.

20. The method of claim 17 wherein said pharmacokinetic modulator is a liposome.

21. The method of claim 17 wherein said reactive moiety is selected from the group consisting of a hexylamine, diene or dienophile.

22. The method of claim 14 wherein said guanosine derivatized at the 5'-positition of the ribose ring is selected from the group consisting of GAP, GAP-

fluorescein, GAP-biotin, GAP-Tc chelate, GAP-TEG, GAP-TEG-fluorescein, GAP-TEG-biotin and GAP-TEG-Tc chelate.

23. The method of claim 14 wherein the RNA polymerase is T7 RNA polymerase.

24. The method of claim 14 wherein said nucleotide triphosphates are modified at the 2'-position of the ribose ring.

25. The method of claim 14 wherein said nucleotide triphosphates are modified at the 5-position of the base.

26. The method of claim 14 wherein the RNA polymerase is T7 RNA polymerase.

27. The method of claim 14 wherein said DNA template comprises a random region and a fixed region.

28. The method of claim 14 wherein said target is selected from the group consisting of a protein, peptide, tissue, carbohydrate, polysaccharide, glycoprotein, hormone, receptor, antigen, antibody, co-factor, inhibitor, drug, dye, nutrient and growth factor.

29. The method of claim 28 wherein said tissue is selected from the group consisting of connective tissue, epithelium, muscle tissue, nerve tissue, organs, tumors, lymph nodes, arteries and cells.

30. The method of claim 14 wherein said molecular entity is selected for use as a therapeutic agent, diagnostic agent, or for use in radiotherapy.

31. The method of claim 14 wherein said molecular entity is selected to improve pharmokinetic behavior, to increase hydrophobicity, to enhance binding, to enhance membrane partitioning and to enhance permeability.

32. A product formed by the method of claim 14.

33. A method of synthesizing bioconjugates comprising:

(a) providing a DNA template;

(b) combining the DNA template with nucleotide triphosphates, a 5'-substituted guanosine, wherein said 5'-substituent contains a reactive moiety and an RNA polymerase under conditions suitable for transcription; and

(c) reacting the product from step (b) with a molecular entity containing a moiety capable of reacting with the reactive moiety on said 5'-substituent.

34. The method of claim 33 wherein said reactive moiety is selected from the group consisting of: amines, dienes, dienophiles, thiols, vinylsulfones, photoaffinity labels and interchelators.

35. The method of claim 33 wherein said RNA polymerase is T7 RNA polymerase.

36. The method of claim 33 wherein said molecular entity is selected for use as a therapeutic agent, diagnostic agent, or for use in radiotherapy.

37. The method of claim 33 wherein said molecular entity is selected to improve pharmokinetic behavior, to increase hydrophobicity, to enhance binding, to enhance membrane partitioning and to enhance permeability.

38. A product formed by the method of claim 33.

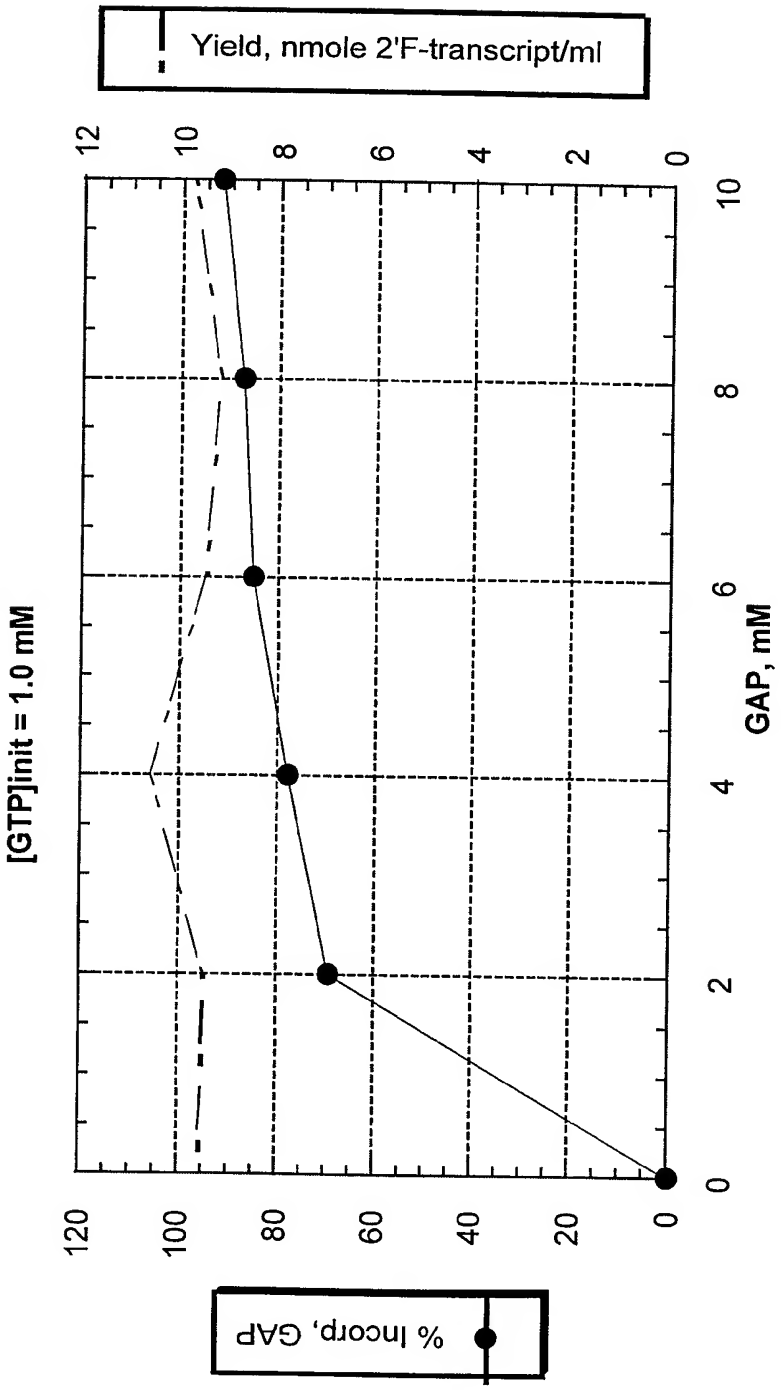

Yield, nmole 2'F-transcript/ml
[GTP]init = 1.0 mM
GAP, mM
% Incorp, GAP
0
2
4
6
8
10
12
20
40
60
80
100
120

Fig. 1

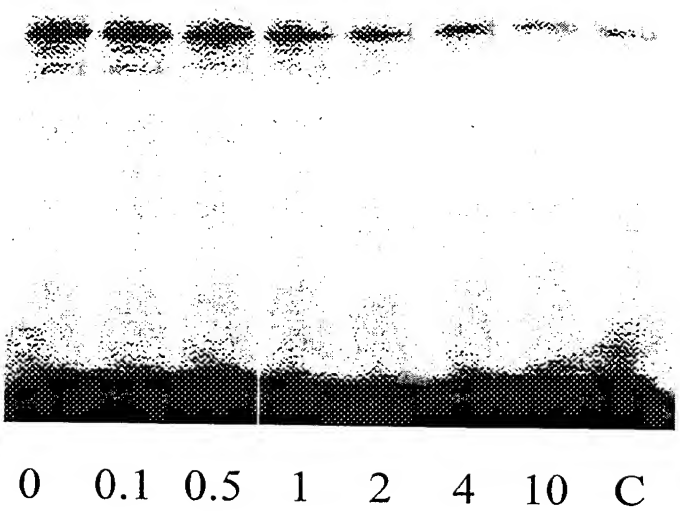


Fig. 2

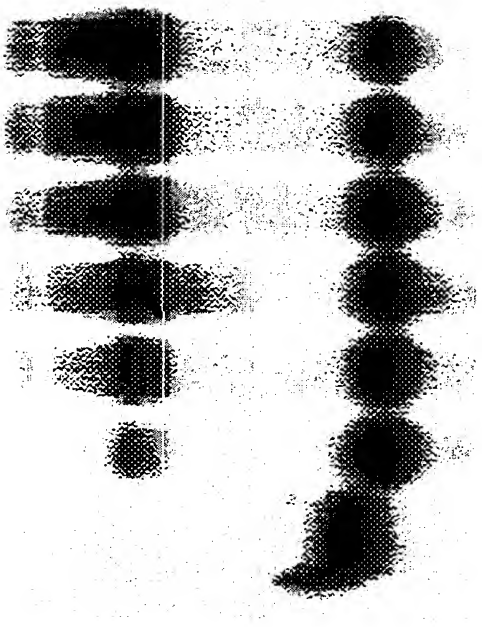


Fig. 3

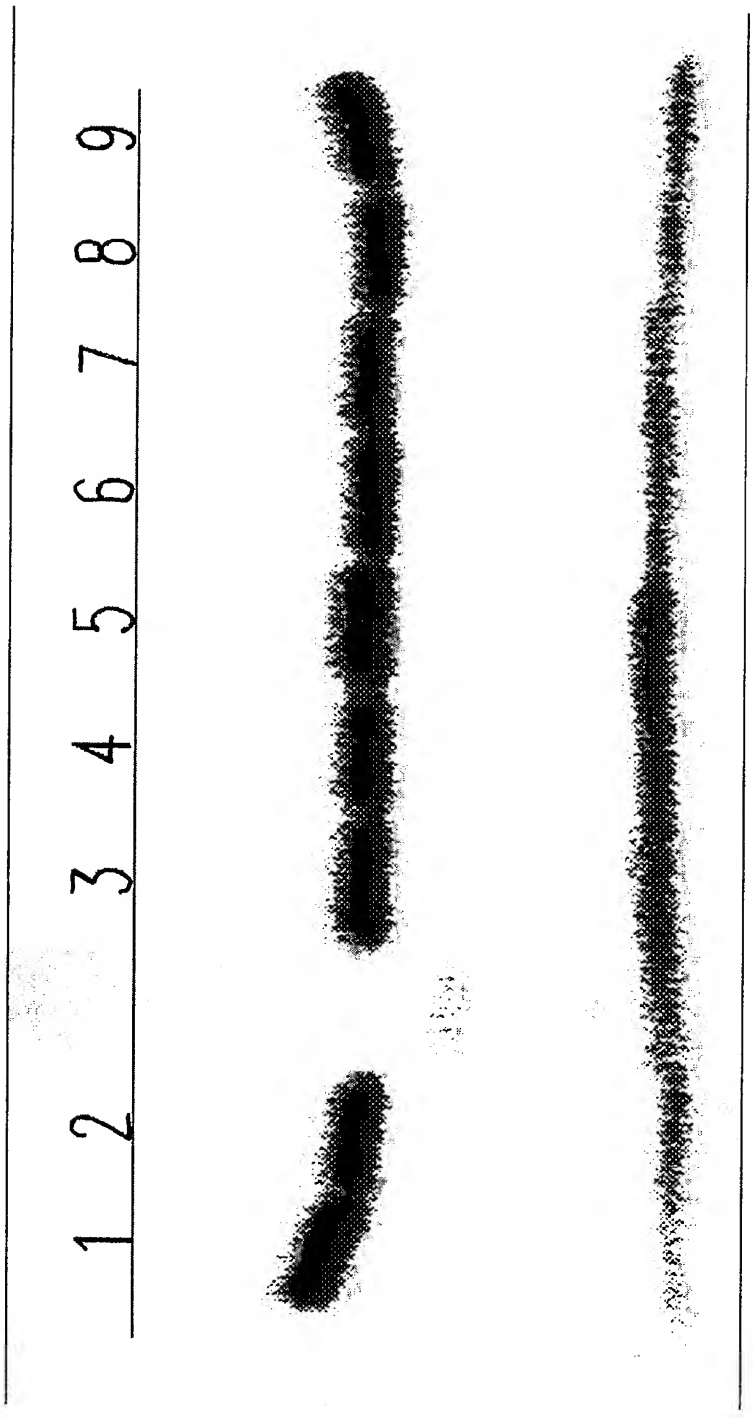


Fig. 4A

8 9

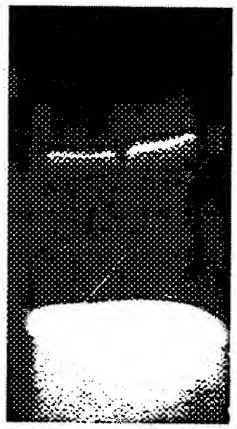


Fig. 4B

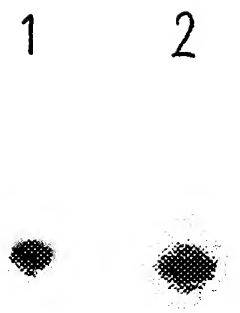


Fig 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/00589

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12P 19/34; C07H 21/02
US CL :435/6, 91.2; 536/23.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.2; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE, BIOSIS
search terms, RNA polymerase, transcription, 5' modified guanosine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LOHSE, P.A. et al. Ribozyme-catalysed amino-acid transfer reactions. NATURE. 30 May 1996. Vol. 381, pages 442-444, see entire document.	1-38
Y,E	US 5,712,375 A (JENSEN et al.) 27 JUNE 1998, see entire document.	1-38
Y,P	US 5,688,935 A (STEPHENS et al.) 18 November 1997, see entire document.	1-35

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 25 MARCH 1998	Date of mailing of the international search report 06 MAY 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Lisa Arthur Telephone No. (703) 308-0196

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